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Scavenging of reactive oxygen species by a novel glucurinated flavonoid antioxidant isolated and purified from spinach

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Abstract

NAO is a natural water soluble antioxidant that was isolated and purified from spinach leaves. Using HPLC, NMR, and CMR spectroscopy, the main components were identified as flavonoids and *p*-coumaric acid derivatives. The NAO was found to be a very effective antioxidant in several in vivo and in vitro biological systems. In the present study, the antioxidant activity of the novel antioxidant glucurinated flavonoid (GF) isolated and characterized from NAO, is compared to well-known antioxidants. In addition, the direct free radical scavenging properties of the purified component GF were studied using the electron spin resonance (ESR) technique. GF and NAO were found to be superior to EGCG and NAC and to the Vitamin E homologue Trolox in inhibiting reactive oxygen species (ROS) formation in the autooxidation system of linoleic acid and in fibroblasts exposed to metal oxidation. GF and NAO were found to inhibit the ESR signal intensity of DMPO-O_2 radical formation during the riboflavin photodynamic reaction. 10 mM GF caused approximately 90% inhibition in the intensity of the ESR signal, while NAO at a concentration of 60 μg/ml caused an inhibition of about 50%. Using the Fenton reaction, GF and NAO were found to inhibit DMPO-OH radical formation. A concentration of 2 mM GF caused a 70% inhibition in the intensity of the DMPO-OH radical ESR signal, while propyl gallate at the same concentration caused only 50% inhibition. Furthermore, both GF and NAO also inhibited the $^1O_2$ dependent TEMPO radical generated in the photoradiation TPPS4 system. About 80% inhibition was obtained by 4 mM GF. The results obtained indicate that the natural antioxidants derived from spinach may directly affect the scavenging of ROS and, as a consequence, may be considered as effective sources for combating oxidative damage.

Keywords: Natural antioxidant (NAO); GF; Spinach; ESR; ROS

1. Introduction

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various diseases as well as in the normal process of aging (Aust et al., 1993; Stohs, 1995). The reactive oxygen species (ROS) formed during normal metabolic processes can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. Free radical scavengers and antioxidants can reduce lipid peroxidation and the generation of reactive oxygen species. The importance of antioxidants in human health has become increasingly clear due to spectacular advances in understanding the mechanisms of their reaction with oxidants. Furthermore, some human epidemiological studies have shown that natural antioxidants have potential health benefits (Block, 1992). Most human studies have been performed with well-established substances like Vitamin E, Vitamin C, β-carotene, or trace metals like selenium (Noda et al., 1997). However, there is increasing interest in the antioxidant activity of the phytochemicals present in our diet, in health food supplements (neutraceuticals), and in topical preparations that protect the skin from environmental exposure. The protection that fruits and vegetables provide against diseases, including cancer and cardio- and cerebrovascular diseases, has been attributed to the various antioxidants contained in them (Boveris and Puntarulo, 1998).

We have reported the presence of a series of powerful water soluble natural antioxidants in spinach leaves and have described their potential biological activity (Grossman et al., 1994; Zurovsky et al., 1994, 1995;

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Zurovsky and Gispaan, 1995; Ben-Shaul et al., 2000; Lomnitski et al., 2000a,b,c; Nyska et al., 2001). Recently, we described for the first time the isolation and chemical identification of several of these antioxidant components. Four components were identified as glucuronic acid derivatives of flavonoids, three components as *trans* and *cis* isomers of *p*-coumaric acid, and other components as *meso*-tartarate derivatives of *p*-coumaric acid (Bergman et al., 2001).

Since no systematic study has been reported on the direct action of these substances on active oxygen species, we used electron spin resonance (ESR) spectroscopy with spin trapping to get evidence for the reduction of radical intermediates by the novel GF, obtained by HPLC purification of NAO, as compared to other known antioxidants.

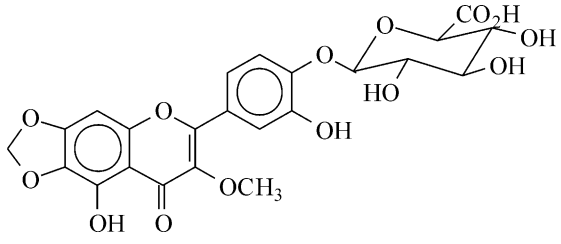
In a recent publication, Noguchi et al. (2001) discussed the diverse function of antioxidants, classifying them by function into four categories: preventive antioxidants; radical scavenging antioxidants; repair and de novo antioxidants; and adaptation. They claim that radical scavenging antioxidants have the greatest advantage and their chemical structure is of key importance.

In the present study, we elucidate that the novel natural antioxidant GF of spinach is an efficient antioxidant and a free radical scavenger.

2. Results

2.1. Effect of antioxidants in autooxidation system

One of the main components of NAO isolated and characterized is the glucurinated flavonoid (GF), having the chemical structure of 6-(3,4-dihydroxy-phenyl)-9-hydroxy-7-methoxy-[1,3]dioxolo[4,5-g]chromen-8-one 4′-β-glucuronid, shown in the following scheme.



The antioxidant activity of NAO, isolated from spinach, and that of the GF in comparison to other known antioxidants, is presented in Fig. 1, using the system of the autooxidation of linoleic acid.

The inhibition of linoleic acid oxidation was studied with various concentrations of antioxidants as time dependent of preventive activity of autooxidation of linoleic acid (Fig. 1). The selected concentration of each of the antioxidant tested was chosen based on its inhibitory activity after 12 h of incubation. Similar inhibition levels were chosen for the various antioxidants. The representative results are demonstrated in Fig. 1. Both NAO and GF were superior to NAC and EGCG from green tea in protecting linoleic acid against oxidation. While the protection of NAO and GF against the peroxidation of linoleic acid lasted at least for 72 h, the protection of NAC and EGCG lasted only for 24 and 48 h, respectively. Trolox exhibited strong antioxidant activity similar to that obtained from the GF of spinach. However, these results were obtained with a concentration of Trolox that was five times higher than the concentration of GF (Fig. 1).

2.2. Inhibition of ROS in growing cells (fibroblasts) by GF and other antioxidants

Using the DCFH-DA fluorescent assay, the production of ROS by 3T3 fibroblasts in response to metals such as iron was monitored. The IC_{50} of several antioxidants, including GF and NAO on oxidative products measured 1 h after exposure to iron, is described in Table 1. Exposing the fibroblasts to Fe^{++} resulted in a significant increase in ROS. However, adding antioxidants, such as Trolox, EGCG (from green tea), NAC, NAO, and GF, to the incubation medium prior to exposure to Fe^{++} significantly inhibited the oxidative response in the cells (Table 1). EGCG, GF, and NAO were superior to NAC in protecting against ROS production.

In the investigations presented here, the scavenging activity of the natural antioxidants (NAO) isolated from spinach and the purified component GF were monitored.

2.3. Effect of GF on superoxide anion radical

The ability of GF and NAO to scavenge the superoxide free radical was monitored in two different systems. In Fig. 2, the spectrum of DMPO–OOH generated in the xanthine–xanthine oxidase system is

Table 1
Inhibition of ROS formation by antioxidants in 3T3 fibroblasts exposed to Fe^{++} oxidation

Antioxidant	IC_{50} (μM)
GF	0.8
NAC	30.0
Trolox	8.0
EGCG	0.7
NAO	4.0 μg/ml[a]

The level of ROS in the tissue culture was determined using DCFH-DA. The conditions are described in Materials and methods.

[a] The concentration of NAO was given in μg because the molecular weight is unknown.

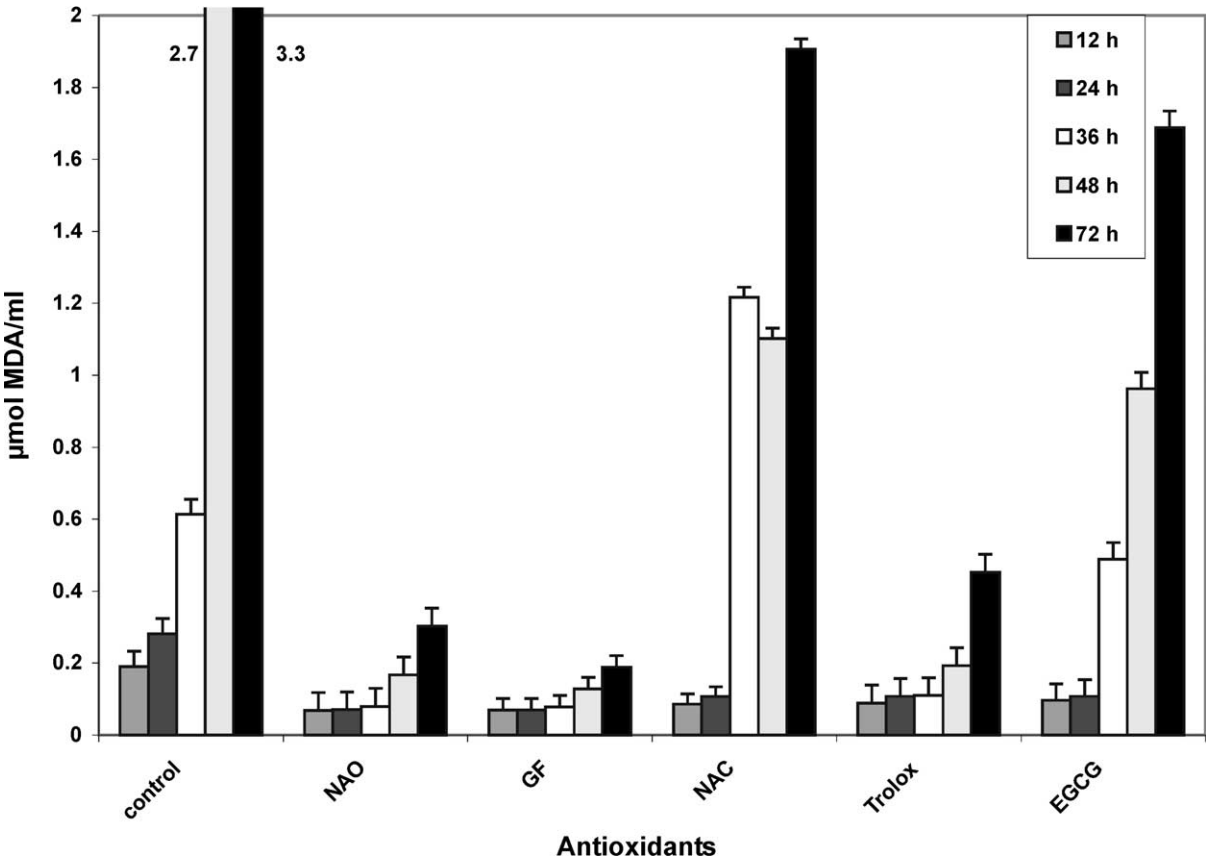


Fig. 1. Time-dependent curve for the inhibition of autooxidation of linoleic acid by antioxidants. Linoleic acid was exposed to autooxidation at 37 °C and MDA values were determined by the reaction with the chromogenic reagent *N*-methyl-2-phenylindole.The chromophore absorbs at 586 nm. The concentrations of the antioxidants in the reaction mixture: NAO 5 μg; GF 2 μM; NAC 10 μM; Trolox 10 μM; EGCG 2 μM.

presented. It shows a typical ESR spectrum resulting from the interactions of an uncoupled electron with a primary atom as well as with the secondary beta and gamma protons. At 10 mM GF, about 90% inhibition in the intensity of the ESR signal was obtained [Fig. 2(c)]. 30 μg NAO exhibited about 50% inhibition [Fig. 2(b)]. It also should be pointed out that adding SOD (2 units) eliminated the ESR spectrum of the OOH adduct (not shown).

The ability of both GF and NAO to scavenge the superoxide free radical was also assayed in the irradiated riboflavin/EDTA system. A typical ESR spectrum of the DMPO–OOH spin adduct was detected as shown in Fig. 3(c). The kinetics of the build-up in the superoxide radical ESR signal is shown in Fig. 3(a), in which the rate of superoxide radical formation is proportional to irradiation time. When irradiation was terminated, the ESR signal intensity decreased with time. Upon irradiation in the presence of GF, the initial rate of the ESR signal increased with increasing irradiation time, but with a lower intensity in ESR signal compared with Fig. 3(a). NAO, like GF, significantly inhibited the intensity of the ESR signal (not shown).

2.4. Effect of GF on hydroxyl radical

The Fenton reaction, a well known •OH generator, was used to test the ability of GF and NAO to scavenge this free radical. The typical ESR spectra of the spin adduct of DMPO–OH observed in the Fenton reaction is shown in Fig. 4(a). In the presence of 2 mM of the flavonoid component, the concentration of DMPO–OH decreased by about 60% [Fig. 4(b)]. NAO demonstrated a similar inhibitory effect (not shown). The widely used antioxidant propyl-gallate was also assayed in this system, and it exhibited scavenging activity toward the •OH [Fig. 4(c)]. Using a similar concentration (2 mM) for both GF and propyl gallate, the effect of the spinach antioxidant in scavenging the •OH was stronger. Both antioxidants were tested at final pH of 7.4.

2.5. Effect of GF on singlet oxygen

A typical ESR spectra of the spin adduct of singlet oxygen is demonstrated in Fig. 5(a). Four mM GF inhibited the intensity of the signal by about 70% [Fig. 5(b)].

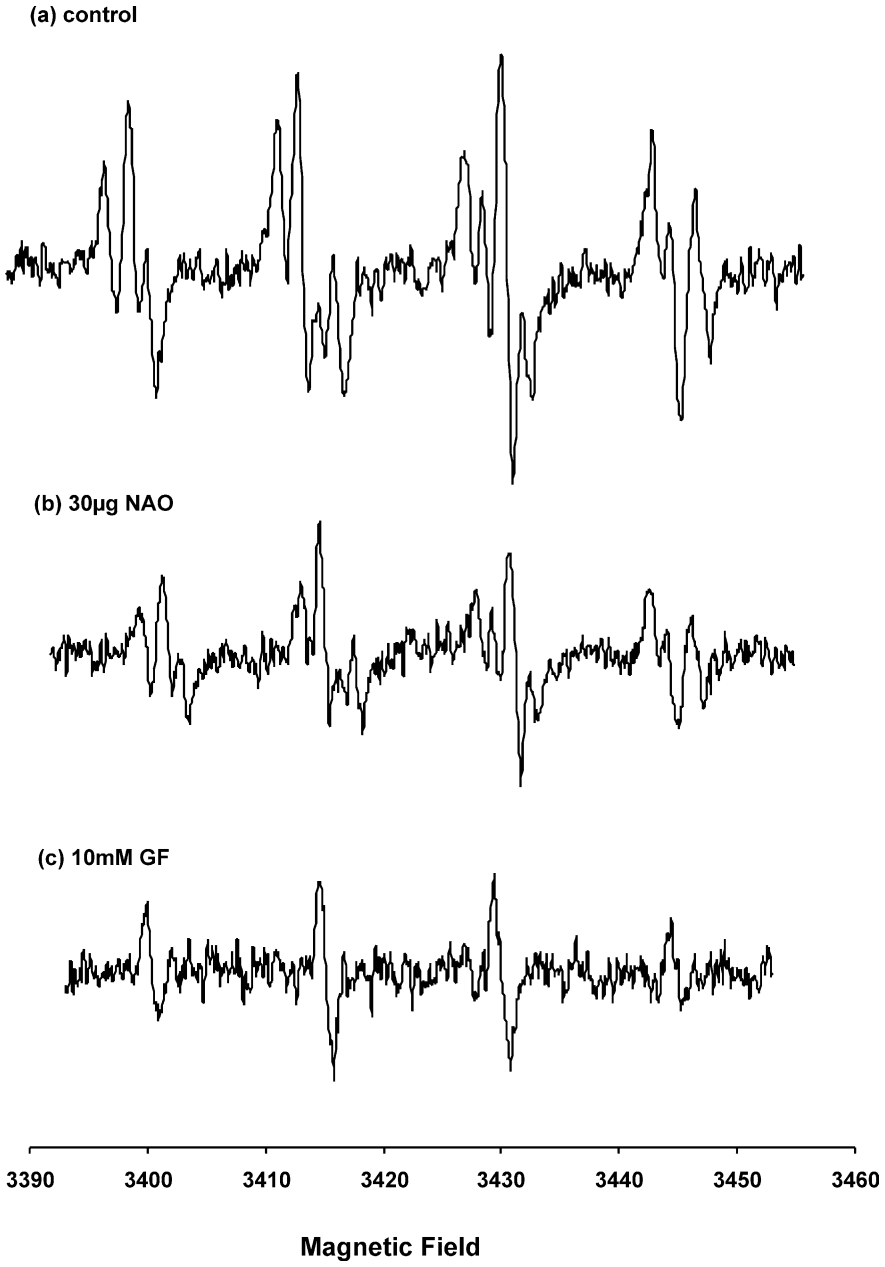


Fig. 2. ESR spectra of the spin adduct of superoxide radical observed during the reaction of xhanthine (20 μM) and xanthine oxidase (0.015 U) containing 200 mM DMPO and 0.2 mM DPTA in air-saturated PBS (pH 7.4), in the absence and presence of GF. (a) Control, containing xanthine, xhanthine oxidase, DMPO, DPTA and PBS. (b) As in (a) in the presence of 30 μg NAO. (c) As in (a) in the presence of 10 mM GF.

3. Discussion

Recently, we reported the identification of the chemical structure of the main components of the natural water-soluble antioxidants (NAO) isolated from spinach (Bergman et al., 2001). The two main components were identified as flavonoids and *p*-coumaric acid derivatives. The present study was undertaken to elucidate the free radical scavenging activity of both the novel GF purified antioxidant and the NAO of spinach. In the investigation presented here, special interest was given to the flavonoid component, GF. We compared the antioxidant activity of the purified components derived from NAO with other well-established antioxidants. It is clear from the data obtained that the natural antioxidants isolated from spinach are very efficient antioxidants compared to the commercially known antioxidants (Fig. 1). The efficacy of the the purified antioxidant component GF and NAO was also elucidated in a tissue culture system (3T3 fibroblasts) as being superior to Trolox and NAC in reducing the level of ROS induced by metal oxidation (Table 1).

Using ESR spectroscopy, we showed for the first time in a direct assay that NAO and GF are free radical

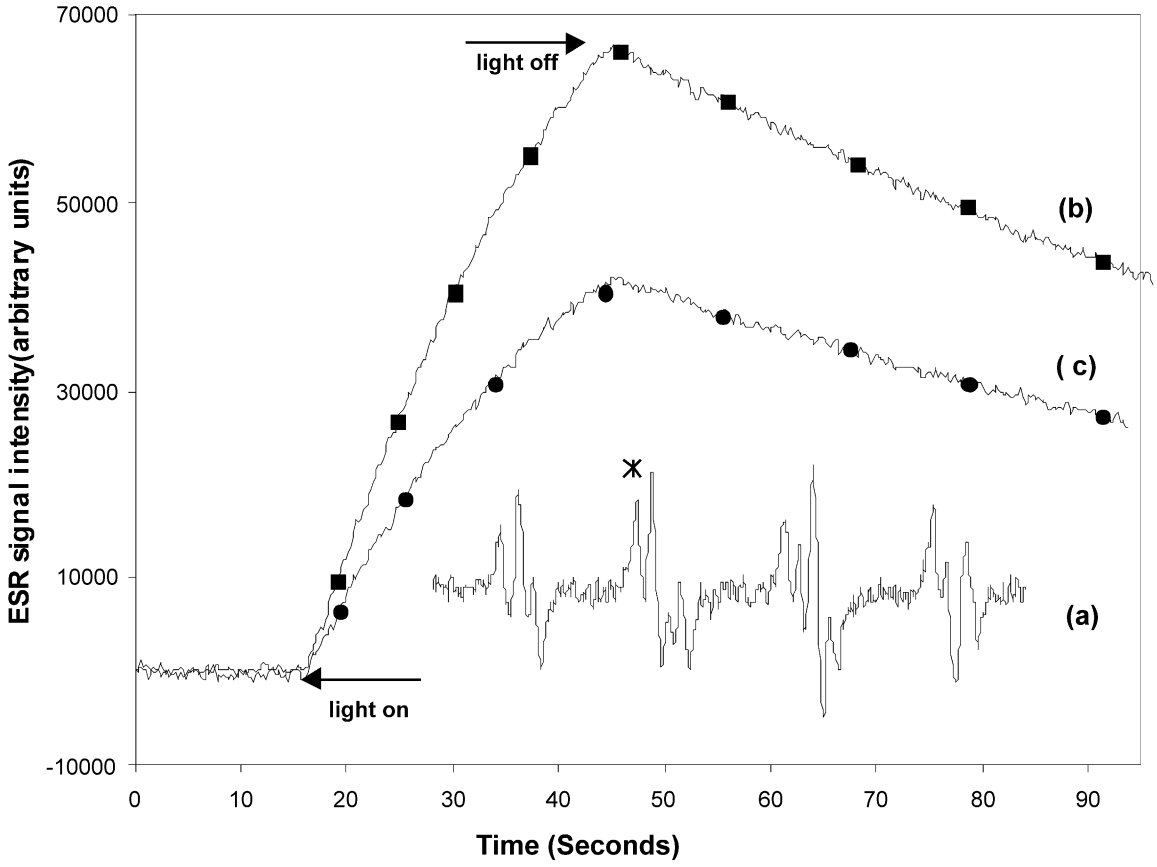


Fig. 3. Effect of GF on the ESR spectra of DMPO radical adducts obtained in the irradiated riboflavin system. (a) The ESR spectra of DMPO radical adducts. The spectra were obtained after 30 s irradiation of the sample containing 0.3 mM riboflavin, 5 mM EDTA, and 0.1 M DMPO in PBS (pH 7.4). The * marks the superoxide adduct. (b) Time-dependent kinetic of the appearance of the DMPO–OOH adduct after irradiation (light on) and the decrease of the adduct after termination of the irradiation (light off). (c) As in (b) in the presence of 0.4 mM GF.

scavengers. In the present study, the ability of NAO and GF to inhibit ROS generation was related to their ability to scavenge $^{\bullet}$OOH, $^{\bullet}$OH, and $^1O_2$ free radicals.

This broad spectrum of antioxidant activity is not characteristic of all natural antioxidants that have been studied. Boveris and Puntarulo (1998) tested the potential of wheat, alfalfa, and ginko biloba extracts on scavenging free radicals resulting from lipid peroxidation in rat liver microsomes. While ginko biloba extract was efficient in scavenging the $^{\bullet}$OOH formed in the microsomes, both wheat and alfalfa extracts were not able to inhibit the superoxide free radicals. Noda et al. (1997) measured the free radical scavenging activities of water-soluble extracts from some natural sources. They found that ginko biloba extract, β-catechin, and green tea were efficient in scavenging both superoxide and hydroxy free radicals. The antioxidative effects of a processed grain food which contained extracts of germ, soybean, rice bean, tear grass, sesame, wheat, citron, green tea, green leaf, and malted rice was studied by Minamiyama et al. (1994); the food mixture significantly inhibited the DMPO–OOH and DMPO–OH signals. Yamaguchi et al. (1999) examined the scavenging effect of grape seed extract using the ESR technique and reported that it showed strong scavenging activity on superoxide radicals and weak activity on hydroxyl radicals.

Shi et al. (2000) reported that EGCG is capable of scavenging both $^{\bullet}$OOH and $^{\bullet}$OH. On the other hand, baicaein and baicalin isolated from *Scutellaria rvularis* demonstrated a strong activity in eliminating superoxide radicals but no significant effect on scavenging hydroxyl radicals (Shieh et al., 2000). Masaki et al. (1993) reported that hamamelitannin, which is a component of the bark extract of hamamelis (*Hamamelis vrginior* L.), was found to be a potent scavenger of superoxide anion radicals and protected fibroblasts from peroxidation. This review shows that there are significant differences in the ability of various natural plant sources in scavenging free radicals.

The data presented in the current study also indicate the superiority of the natural antioxidants of spinach over well-established antioxidants, such as NAC, Trolox, and propyl gallate, in the studied systems. This superiority was demonstrated both in the ability of the antioxidants to inhibit ROS formation (Fig. 1 and Table 1) and in its broad free radical scavenging activity (Figs. 4 and 5) The superiority of GF over propyl

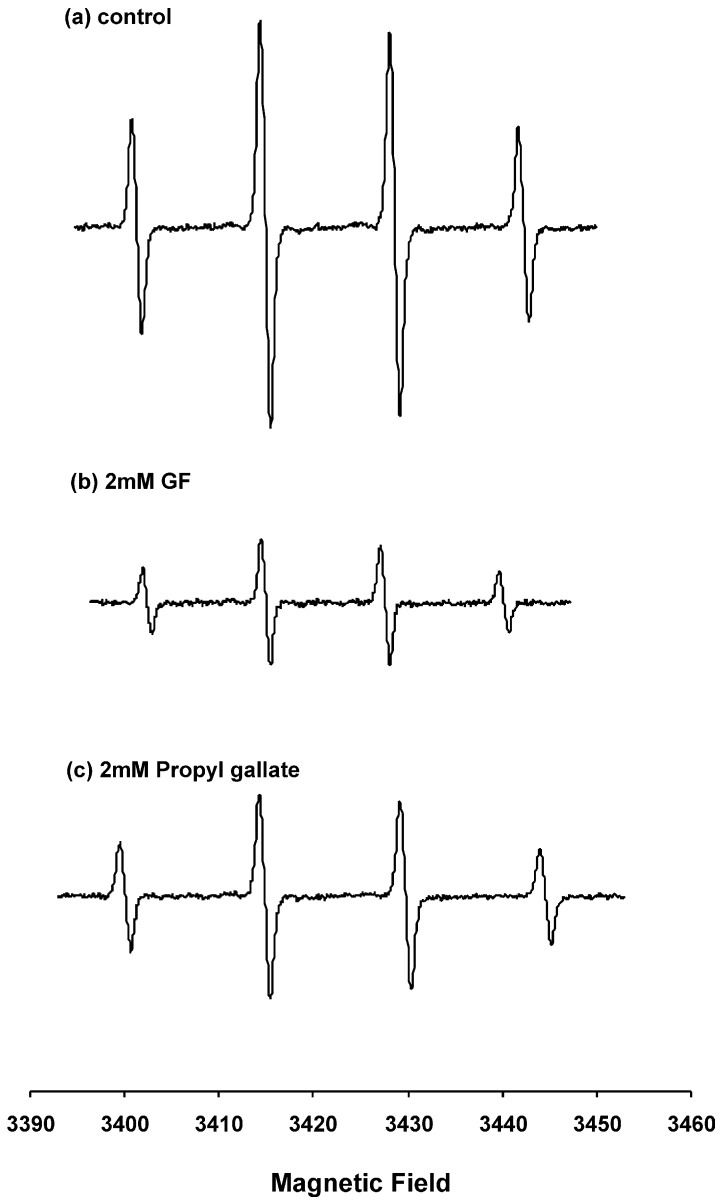


Fig. 4. Hydroxyl radical scavenging activity of the antioxidants GF and propyl gallate. (a) ESR spectrum of DMPO–OH generated by a Fenton-type reaction (10 mM DMPO, 100 μM $FeSO_4$, 10 mM H_2O_2, in a final volume of 0.5 ml. (b) As in (a) in the presence of 2 mM GF. (c) As in (a) in the presence of 2 mM propyl gallate.

gallate in the hydroxyl radical scavenging assay, pH 7.4 (Fig. 4) may be explained by the better polarity of GF. This is in accordance with the finding described by HaselofF et al. (1990) concerning the hydroxyl radical scavenging activity of a number of lipophilic and hydrophilic benzoic acid derivatives. Yoshimura et al. (1999) compared the activity of two well established antioxidants, tocopherol and ascorbic acid, in the H_2O_2/NaOH/DMSO system, which simultaneously forms a superoxide anion, hydroxyl radical, and methyl radical. They found that ascorbic acid specially inhibited the superoxide anion and hydroxyl radical, whereas tocopherol suppressed the methyl radical. The superoxide radical scavenging activity of phenolic compounds was reported by Tsujimoto et al. (1993).

Guo et al. (1999) examined the relationship between the free radical scavenging activities and the chemical structures of tea catechins toward superoxide free radicals and singlet oxygen using ESR techniques. They suggested that the presence of the gallate group at the 3 position of the catechins plays the most important role in their free radical-scavenging abilities and that the additional insertion of the hydroxyl group at the 5′ position in the B ring also contributes to their scavenging activities. The GF described in the present study contains a hydroxyl group at the 5′ position in the B

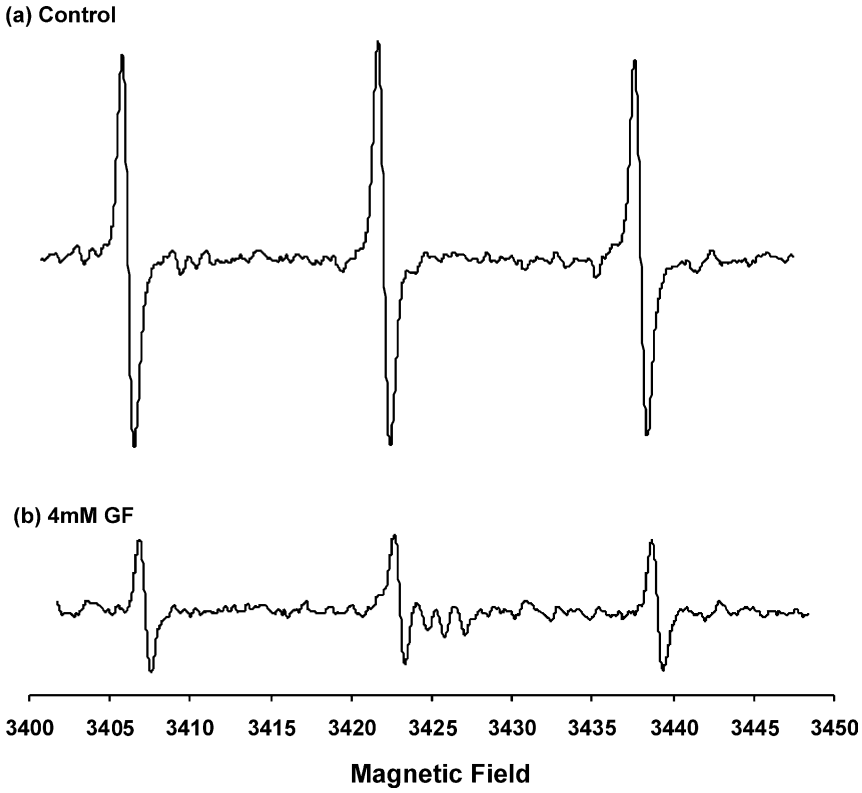


Fig. 5. Effect of GF on the ESR signal of singlet oxygen. (a) The ESR signal of TEMP radical. Singlet oxygen was generated in the photoradiation-porphine system. The reaction mixture contained 0.054 mM 5,10,15,20,-tetra (4-sulfonatophenyl) porphine ($TPPS_4$), 30 mM TEMP, and 50 mM of PBS (pH 7.4) was present in the control. (b) As in (a) in the presence of 4 mM GF.

ring. Its antioxidant activity was elucidated in both direct (Figs. 2–5) and indirect assays (Fig. 1 and Table 1). The GF isolated from spinach was superior to EGCG in the autooxidation assay (Fig. 1) and exhibited similar antioxidant activity in protecting against ROS formation in fibroblasts (Table 1).

In the present study, we demonstrated that GF and NAO of spinach also scavenge singlet oxygen. Liao et al. (2000) reported that a water soluble extract of *Alpinia speciosa*, which is known for its beneficial effects, exhibited both antioxidant and singlet oxygen scavenging activities.

The broad free radical scavenging property of the natural antioxidants of spinach, described in the present study, may explain the strong antioxidant activity of these components in both in vitro and in vivo models (Grossman et al., 1994; Zurovsky et al., 1994, 1995; Zurovsky and Gispaan, 1995; Ben-Shaul et al., 2000; Lomnitski et al., 2000a,b,c; Bergman et al., 2001; Nyska et al., 2001).

Moreover, it may also explain the strong synergistic effect obtained with the NAO as an antioxidant compared to the activity of isolated fractions derived from the NAO (Bergman et al., 2001). In a similar way, Packer et al. (1999) demonstrated that an extract of pine (*Pinus maritima*) bark which is composed of a mixture of flavonoids, mainly procyanids and phenolic acids, displays greater biologic effects as a mixture than its purified components do individually, thus indicating that the components interact synergistically.

Although not yet shown by a direct ESR study, we may assume that the oligomeric components of NAO may contribute to its strong and broad free radical-scavenging activity against reactive oxygen species. These results suggest that there is promising potential for the natural antioxidants of spinach to be used successfully in treating human diseases that involve free radical and oxidative damage.

4. Experimental

4.1. Materials

TEMPONE (2,2,6,6-tetramethyl-4-piperidone), xanthine, xanthine oxidase, riboflavin, EDTA, PBS, DTPA (Diethylentriaminepentaacetic acid) and $FeSO_4$, were obtained from Sigma Chemical, USA. DMPO was purchased from Calbiochem, CA, USA and hydrogen peroxide (H_2O_2) was obtained from Bio Lab. Trolox was obtained from Acros, USA.

4.2. Antioxidants

Several antioxidants were used in this study. Trolox (Vitamin E homologue) was purchased from Acros Organics, NJ, USA. BHT (butylated hydroxy toluene), propyl gallate and NAC (*N*-acetyl cysteine) were purchased from Sigma Chemical, USA.

NAO, the natural antioxidant of spinach, and the purified flavonoid of NAO were prepared according to Bergman et al. (2001). The HPLC isolated flavonoid fraction (GF) with RT of 21.7 was used as an antioxidant in this study.

4.3. Determination of MDA

The determination of MDA was based on the reaction of the chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals at 45 °C. The chromophore absorbs at 586 nm (Esterbauer et al., 1991).

R-1: A stock of 10 mM solution of *N*-methyl-2-phenylindole in acetonitrile. R-2: Metanesulfonic acid.

For the assay, 96-well plates were used. A sample of 50 μl of the tested material was added to each well, followed by 160 μl of R-1 and 40 μl of R-2. Three replicates were prepared for both controls and samples. A similar procedure was used for the standard; TMP (tetramethoxy propane) used at concentrations from 0.8 to 8 μM. The plate was incubated for 48 min at 45 °C. The microplate was read at 586 nm using a spectrofluorometer (Tecan).

4.4. Determination of ROS in cultured cells

The level of ROS in the cells was assayed according to Rosenkranz et al. (1992). The nonfluorescent 2′,7′-dichloroflorofluorescin-diacetate (DCFH-DA) was used for monitoring the ROS with a spectrofluorometer (wavelength 485/535 nm) capable of reading microplates. 3T3 cells were maintained in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum. After the 3T3 cells had been allowed to grow to 70% confluency, they were detached with a solution of 0.1% trypsin. The cells were washed immediately with PBS and counted. 3T3 cells were counted, plated 10^5/well/per 50 μl PBS in 96-well tissue culture plates (flat-bottomed). Antioxidants at various concentrations (or PBS for the control samples) were added to the cells in wells. A stock solution of DCFH-DA was prepared by dissolving 2.0 mg/ml in ethanol. The stock solution was kept at −20 °C, wrapped in foil in the dark. This stock solution was diluted 100 times in PBS and 100 μL were added to each well in the microplate. Oxidation was initiated by addition of $FeSO_4$ at a final concentration of 80 μM to the wells. All the plates were incubated for 1 h at 37 °C and then read with a Tican fluorometer (wavelength 485/530 nm).

4.5. Determination of antioxidant activity

Antioxidant activity was determined by measuring the inhibition of autooxidation of linoleic acid incubated at 37 °C from 12 h up to 72 h. Samples were drawn from the incubation mixture for oxidation analysis at certain periods. Linoleic acid was prepared in Tween 20 as described by Grossman and Zakut (1978). In brief, a stock solution of 3×10^{-2} M linoleic acid was prepared by addition of 3 nmol of linoleic acid to 50 ml of distilled water containing 1 ml of Tween 20. Three to 5 ml of 1 N NaOH were added in order to clarify the resulting emulsion, and the volume was adjusted to 100 ml with distilled water. This stock solution was diluted with 100 mM phosphate buffer pH 7.0 to 7.5×10^{-3} M. The solution was stored at 4 °C for up to 2 weeks. One ml of this solution was used for the autooxidation assay. It was incubated at 37 °C for certain periods of time in the presence or absence of antioxidants, and from this solution 25–100 μl were taken for MDA assay as described above.

4.6. Recording of ESR signals

The ESR spectra were recorded on a Bruker ER 100d X-band spectrophotometer. The measurements were repeated at least four times for each sample.

After acquisition, the spectra were processed by the Bruker WIN-EPR software version 2.11 for baseline correction, noise filtration and for integrating the signals. (In all the figures the intensity is expressed in arbitrary units).

4.7. Superoxide anion assay

Two sources were used for the production of the superoxide free radical.

The conditions of ESR measurement of the superoxide radical were as follows: microwave frequency, 9.66 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 1 G; and time constant, 0.328 s weep time 167.72 s receiver gain 2×10^5.

4.7.1. The riboflavin/EDTA irradiated system

Superoxide anions were generated by the irradiated riboflavin/EDTA system (Zhao et al., 1989). The reaction mixture contained 0.3 mM riboflavin, 5 mM EDTA, 0.1 M DMPO, and various concentrations of NAO or other antioxidants in 0.05 M pH 7.4 PBS. PBS was present in control. The assay mixture was transferred to a quartz capillary and fitted into the cavity of the Varian ESR spectrometer.

ESR spectra were recorded following irradiation by a 25 W lamp at room temperature. Kinetic spectra were obtained by fixing the magnetic field at 3413 G.

4.7.2. The xanthine–xanthine oxidase system

The reaction of xanthine and xanthine oxidase was used as a source of $O_2^{\cdot-}$(Zang et al., 1995). Prior to performing ESR studies, we examined whether NAO or the purified components inhibit xanthin oxidase activity by monitoring uric acid formation spectrophotometrically at 290 nm. There was no significant change in urate formation, indicating that NAO and its purified components do not inhibit the enzyme. The typical reaction mixture consisted of DMPO (100 mM), xanthine (20 μM), xanthin oxidase (0.015 U), and DPTA (0.2 mM) in air saturated PBS, pH 7.4. The reaction was started by adding xanthine oxidase from a stock solution prepared in PBS, PH 7.4. The assay mixture was transferred to a quartz capillary and fitted into the cavity of the Varian ESR spectrometer.

4.8. The hydroxy free radical assay

The fenton reaction ($Fe^{2-} + H_2O_2 \rightarrow Fe^{3-} + {}^{\bullet}OH + OH^-$) was utilized to test the ability of antioxidants to scavenge $^{\bullet}OH$ (Zang et al., 1998). The reaction mixture contained 10 mM DMPO, 100 μM $FeSO_4$, 10 mM H_2O_2, in the absence and presence of antioxidants in a final volume of 0.5 ml.

The conditions of ESR measurement were as follows: microwave frequency, 9.66 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 0.6 G; and time constant, 0.328 s and seep time 336 s receiver gain 2×10^5.

4.9. Determination of singlet oxygen

Singlet oxygen was generated in the photoradiation-porphine system. The reaction mixture contained 0.054 mM 5,10,15,20,-tetra (4-sulfonatophenyl) porphine ($TPPS_4$), 30 mM TEMP, and antioxidants. The mixture was irradiated by a visible light projector with a 25 W lamp at room temperature for 5 min, and ESR spectra were recorded. The singlet oxygen detection is based on the specific reaction between $^1O_2$ and TEMP which forms a stable and ESR detectable TEMPO radical. The conditions of ESR measurement were as follows: microwave frequency, 9.66 GHz; modulation frequency, 100 kHz; microwave power, 15 mW; modulation amplitude, 1G and time constant, 0.655 s. and seep time 336 s receiver gain 2×10^5.

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